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A High-Yielding Enzymatic Process for Production of Bioactive β-1,3-Glucan Oligosaccharides with Defined Size

Ningzi Guan[1], Juping Yu[2], Wei Liu[2] and Rachel Chen[1*]

[1]*School of Chemical and Biomolecular Engineering, Georgia Institute of Technology, Atlanta, GA 30332-0100, USA*

[2]*Jiangsu Key Laboratory of Druggability of Biopharmaceuticals, China Pharmaceutical University, Nanjing, 210009, PR China*

[*]**Corresponding author:** Rachel Chen, School of Chemical and Biomolecular Engineering, Georgia Institute of Technology, Atlanta, GA 30332-0100, USA, Tel: 1404 8941255; Fax: 404 8942866; E-mail: rchen@chbe.gatech.edu

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Abstract

The study is to develop a bioprocess to depolymerize curdlan for production of β-1,3 oligosaccharides with a uniform size. A codon-optimized Streptomyces laminarinase was expressed in an *E. coli* system, resulting in an enzyme preparation (19.1 U/L) highly active on solid curdlan substrate without any pre-treatment. In a preparative scale reaction at pH 5.5 and 55°C, 90% conversion and a product concentration close to 27 g/L were achieved within 6 hours. The major product was identified as β3-glucan pentasaccharide based on HPLC, TLC, and MS analyses. A single-enzyme based bioprocess was highly efficient in deriving β3-glucan pentasaccharide from solid curdlan. Enzyme to curdlan ratio and reaction time are critical parameters for high-yield production of β3-glucan pentasaccharide from curdlan.

Keywords: β3-glucan; β3-glucan oligosaccharide; Curdlan; Laminarinase; LPHase

Introduction

β-Glucans are multifunctional polysaccharides widely distributed in animals, plants, fungi and bacteria [1]. Beta-1,3-glucan exhibit immunomodulatory and antitumor effects and prebiotic properties [2-4]. Consumption of oat β3-glucans is associated with benefits of reducing cholesterol levels [5]. As such, β3-glucans and β3-glucan oligosaccharides are important bioactive natural products for both fundamental studies and various probiotic and medical applications such as vaccine adjuvants [6,7]. While β-glucosidase can be used in the synthesis of glucan oligosaccharides, the method requires significant efforts to overcome the thermodynamic limitation in order to use hydrolases in the synthesis direction [8]. Alternatively, β3-glucan synthesized in nature could be used as a source for the production of glucan oligosaccharides. Among various glucan sources, bacterial β-glucans are particularly attractive as they are manufactured inexpensively in large scale through mature fermentation technology. Curdlan, a β-1,3 glucan produced commercially by Agrobacterium strain, representing a practical route for bioactive glucan oligosaccharides [9]. However, curdlan is a recalcitrant polymeric substrate with low water solubility, posing significant challenges in aqueous-based bioprocessing. Consequently, researchers relied on pre-treatment to accelerate the depolymerization. For example, heat treatment (90°C for 30 min) was used to increase the digestibility of enzyme of curdlan, resulting in 60% conversion of curdlan into soluble oligosaccharides [10]. Alkaline neutralization method was developed as an alternative to promote subsequent hydrolysis. While both pre-treatment were effective to some extent, the resulting oligosaccharides varies in degree of polymerization (DP) from 2-10, undesirable for structural and functional studies and for most other applications [11,12]. Microwave-assisted thermal hydrolysis also leads to a mix of oligosaccharides [13]. We report here a method of depolymerization of curdlan without the use of any pre-treatment to derive β-1,3 oligosaccharides with a uniform size. We show that the single enzyme based bioprocess is highly effective and converts over 90% solid curdlan to pentasaccharides and achieve a high product concentration to nearly 27 g/L.

Materials and Method

Strains, plasmids and culture conditions

Escherichia coli JM109 were used as the host for gene cloning and protein overexpression. The *lph* gene encoding a laminarin hydrolase from *Streptomyces matensis* was codon optimized and synthesized by General Biosystems (Morrisville, US) [14]. The synthetic gene was cloned into pQE80L between *Bam*HI and *Hind*III sites to form plasmid pQLPH. Recombinant strains were grown at 37°C in Luria-Bertani medium (5 g/L yeast extract, 10 g/L peptone, 10 g/L NaCl) or LB agar plates supplemented with 100 μg/mL ampicillin.

Protein expression

The expression conditions including IPTG concentration, induction temperature, initial cell density, and induction time were optimized. Overnight seed culture was prepared in LB medium at 37°C and transferred to fresh LB at an inoculum concentration of 1% (v/v). The cells were induced by 0.75 mM IPTG when OD600 of the culture reached 0.3-0.4. The culture was incubated at 30°C for 6 additional hours. Cells were harvested by centrifugation (4,000x g, 10 min, 4°C) and lysed with B-PER Reagent (4 mL/g cell pellet). Lysate was centrifuged at 15,000x g for 5 min after incubating for 10-15 min at room temperature. The supernatant was taken as crude enzyme. SDS-PAGE analysis was performed with 12% Mini-PROTEAN TGX Gels (Bio-Rad, USA).

Enzyme activity assay

The enzyme activity assay (1 mL) contained 50 mM sodium citrate buffer (pH 6.0), 1% (w/v) curdlan and an appropriate amount of crude enzyme. The reactions were carried out at 45°C for 120 min. The amount of reducing sugars released to the supernatant was determined by 3, 5-dinitrosalicylic acid (DNS) method as follows. 100 µL of the reaction supernatant was mixed with 100 µL of DNS solution (0.4 mol/L NaOH containing per liter 10 g of DNS, 300 g of sodium potassium tartrate) [15]. The mixture was then heated at 99°C for 10 min, followed by cooling on ice for 5 min and dilution with 800 µL deionized water before measuring the absorbance at 540 nm. Glucose (0.2-1 mg/mL) was used as standards. One unit was defined as the amount of enzyme releasing 1 µmol of reducing sugar per minute at 45°C and pH 6.0.

Effect of pH and temperature on curdlan hydrolysis

To investigate the optimum reaction condition, hydrolysis was performed in reaction mixture contained 50 mM sodium citrate buffer (pH 5.0-6.5), 1% (w/v) curdlan and 20 mU crude enzyme. The reaction mixtures were incubated at 50-65°C for 60 min. The amount of reducing sugars generated was determined by DNS method as described above. The effect of pH and temperature on curdlan hydrolysis was calculated based on the amount of reducing sugars generated.

Analytic methods

Thin-Layer Chromatography (TLC) analysis was performed to detect the oligosaccharides generated. 2 µL samples were spotted onto HPTLC silica gel 60 plate (Merck KGaA, Darmstadt, Germany), and developed in butanol-acetic acid-water (2:2:1) v/v. The plates were sprayed with orcinol solution (0.2 g orcinol per 75 mL 2.3 M H_2SO_4), and heated at 105°C for 10 min.

The hydrolysates of curdlan were analyzed by HPLC (Agilent 1100) equipped with an Aminex HPX-87H column (300 × 7.8 mm, BIO-RAD, USA) and a differential refraction detector. The samples were centrifuged at 13,000 rpm for 5 min to remove the unreacted curdlan and the supernatant was filtered with a filtration membrane (0.22 µm pore size). H_2SO_4 (5 mM) was used as the mobile phase at a flow rate of 0.3 mL/min.

The oligosaccharide generated from curdlan was identified by ESI-MS at a Thermo LCQ-Deca. The sample diluted in H_2O was introduced by a syringe pump (Harvard Apparatus) and analyzed in negative ionization mode. The electrospray source was set to 3 KV and 150°C, and the automatic gain control was set to 1×10^7 for full scan MS. MS data were acquired and processed using Xcalibur 1.3.

Results and Discussion

Recombinant enzyme preparation

A codon-optimized synthetic gene, *lph* (encoding a laminarinase), was cloned into an *E. coli* T5 promoter expression system pQE80L. Conditions for recombinant protein expression from the transformant JM109/pQELPH were investigated, which include Induction conditions such as initial OD_{600} (0.3, 0.6 or 0.9), IPTG concentration (0.5, 0.75 or 1 mM), induction temperature (25, 30 or 37°C) and induction time (6, 12 or 24 h). As shown in Supplementary Table 1 and SDS-PAGE analysis (Figure 1A), 0.75 mM IPTG concentration, induction at OD_{600} 0.3 and six hours post induction incubation gave the best yield of recombinant enzyme, 19.1 Units/L as measured by an enzyme assay using solid curdlan as the substrate without any pre-treatment (Detailed in Materials and Methods). Recombinant enzyme was prepared after cell lysis and removal of cell debris by centrifuge and used as the crude enzyme. As the present study focused research toward a practical and cost effective bioprocess, only crude enzyme was used.

pH	Temperature (°C)	Reducing sugar (µmol/mL)
5	45	2.47 ± 0.08
5	50	3.95 ± 0.28
5	55	4.28 ± 0.22
5.5	45	3.17 ± 0.23
5.5	50	4.80 ± 0.24
5.5	55	6.48 ± 0.14
6	45	2.95 ± 0.18
6	50	3.84 ± 0.19
6	55	5.23 ± 0.27
6.5	45	3.12 ± 0.22
6.5	50	3.14 ± 0.11
6.5	55	4.05 ± 0.18

Table 1: The effect of pH and temperature on curdlan hydrolysis.

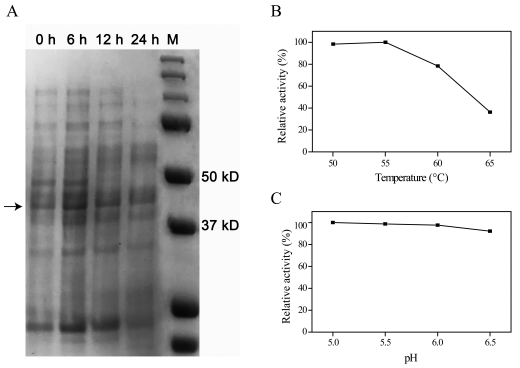

Figure 1: A: SDS-PAGE of cell lysate obtained from different induction time; B: Thermal stability of recombinant lamanarinase; C: pH stability of recombinant lamanarinase.

Impact of pH and temperature on hydrolysis

Initially, a quick 1 h screening of pH and temperature conditions for hydrolysis was conducted. As shown in Table 1, a combination of pH 5.5 and temperature of 55°C gave the highest amount of soluble oligosaccharides from solid curdlan as measured by the total reducing sugar released. It appears that the reaction rate is increased with the increase of temperature. To ensure that enzyme hydrolysis is operated within the range of its stability, the pH and thermal stability of LPHase

were subsequently investigated. As shown in Figure 1B and 1C, 22% and 64% LPHase activity were lost after incubated at 60°C or 65°C for 60 min, while the enzyme was stable over the pH range of 5.0-6.0. Taken together, pH 5.5 and temperature of 55°C are suitable conditions for the hydrolysis. The optimal pH 5.5 was lower than the 6.0 reported by Woo et al. [16]. This may be due to the minor sequence difference of the enzyme and conditions used for analysis (curdlan versus laminarin).

β-glucan pentasaccharide production

To prepare β-1,3 pentasaccharide from curdlan, a scaled-up reaction (10 mL) was run under the optimal conditions identified above. As shown in Figure 2A for substrate concentration of 1% (w/v), pentasaccharides were rapidly accumulated initially, with nearly 70% conversion within two hours. This was followed by a slow rate period until about 10 hours to reach a plateau (90% conversion). In another reaction with 3% (w/v) curdlan, similar trend was observed (Figure 2B). Within the first two hours, the conversion was about 83.6%, suggesting a very rapid reaction. The concentration of product reached a maximum at 6 h and about 26.6 g/L pentasaccharide was generated from 30 g/L curdlan. The conversion at both substrate concentrations were close to 90%, significantly higher than the conversion (60%) reported in a previous study using an enzyme in the same family KfGH64 [10]. Our conversion is slightly higher than that of hydrothermal hydrolysis of curdlan, in which a mixture of β-1,3-glucooligosaccharides were obtained [15].

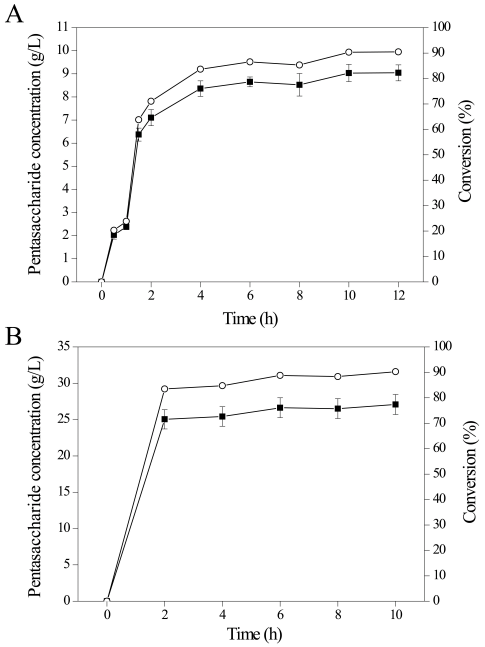

Figure 2: Time profile of curdlan hydrolysis at different substrate concentrations 1% (w/v) (A) and 3% (w/v) (B) curdlan in a 10 mL system. Solid Square, pentasaccharide concentration; hollow cycle, conversion.

To identify the hydrolysis products, supernatant of reaction mixtures were collected after removal unreacted curdlan and proteins. The products were analyzed by HPLC (Figure 3A) and TLC (Figure 3C, Lane 1). As shown in Figure 3A, only one large peak (besides the citrate buffer) was observed on a HPLC spectrum, suggesting that only one major product was produced. This result is corroborated with the TLC (Figure 3C, lane1) which shows only one prominent oligosaccharide spot. For molecular weight analysis, the major product (identified as above) was further purified using activated carbon column and then eluted with 40% ethanol. After freeze-drying, the major hydrolysis product was analyzed by MS. As shown in Figure 3B, the dominate peak corresponds to an M/Z ratio of 827.368, which is consistent with the molecular weight (828.718) of a gluco-pentasaccharide. Combined with the structure of the substrate-a neutral linear β-1,3 glucan, the product was identified as 3 glucan pentasaccharide.

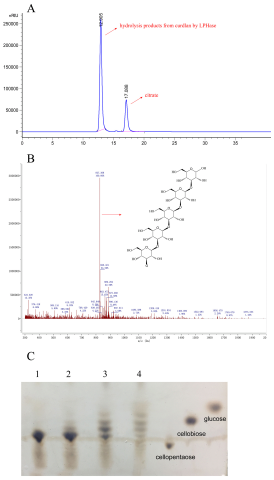

Figure 3: A: HPLC spectrum of the hydrolytic products of curdlan; B: MS analysis of hydrolytic products of curdlan by LPHase; C: TLC analysis of hydrolytic products of curdlan by LPHase. Lane 1, hydrolytic products of curdlan by LPHase when curdlan was abundant; Lanes 2-4, hydrolytic products of laminaripenaose by LPHase for 4, 8 and 12 h, respectively.

Interestingly, while no smaller oligosaccharides were detected by TLC during curdlan hydrolysis, soluble β-1,3 glucan pentasaccharides could be further hydrolyzed by the enzyme. As shown in the TLC (Figure 3C, Lanes 2-4), incubation of enzyme with soluble pentasaccharide led to smaller oligosaccharides. It appears that the enzyme takes off a glucose in a stepwise manner, generating tetra-, tri-, bi-glucose in a sequential manner. At the end of the reaction, when the pentasaccahride disappears, four distinct bands corresponding to tetra, tri, bi and glucose were evident for the 12 h sample (Figure 3C, Lane 4). Since oligosaccharides smaller than pentasaccharides were negligible during the phase of rapid accumulation of pentasaccharide, suggesting that the enzyme hydrolysis is a staged process. During the first stage when abundant solid substrate is available, the enzyme is solely engaged with the solid curdlan. Only when solid curdlan is consumed, the enzyme began to act on soluble pentasaccharide to hydrolyze it to smaller units, one sugar at a time. Therefore, in this stage, the enzyme acts like an exo-glucosidase. To avoid product pentasaccharide degradation, ratio of substrate to enzyme, and reaction time are key process parameters.

Conclusion

A codon-optimized Streptomyces lph was expressed in an *E. coli* system, resulting in an enzyme preparation 19.1 U/L active on solid curdlan substrate without any pre-treatment. In a preparative scale reaction at pH5.5 and 55°C, 90% conversion was achieved within 6 h with pentasaccharide as the only product, reaching a product concentration close to 27g/L. The same enzyme could hydrolyze soluble pentasaccharide but not the solid curdlan to smaller oligosaccharides, thus enzyme to curdlan ratio and reaction time are most critical parameters for high-yield production of β3 glucan pentasaccharide from curdlan.

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